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Structure and activity of a bacterial defense-associated 3′-5′ exonuclease

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Abstract

The widespread CBASS (cyclic oligonucleotide-based anti-phage signaling system) immune systems in bacteria protect their hosts from bacteriophage infection by triggering programmed cell death. CBASS systems all encode a cyclic oligonucleotide synthase related to eukaryotic cGAS but use diverse regulators and effector proteins including nucleases, phospholipases, and membrane-disrupting proteins to effect cell death. Cap18 is a predicted 3′-5′ exonuclease associated with hundreds of CBASS systems, whose structure, biochemical activities, and biological roles remain unknown. Here we show that Cap18 is a DEDDh-family exonuclease related to the bacterial exonucleases RNase T and Orn and has nonspecific 3′-5′ DNA exonuclease activity. Cap18 is commonly found in CBASS systems with associated CapW or CapH+CapP transcription factors, suggesting that it may coordinate with these proteins to regulate CBASS transcription in response to DNA damage. These data expand the repertoire of enzymatic activities associated with bacterial CBASS systems and provide new insights into the regulation of these important bacterial immune systems.

KEYWORDS
bacterial immunity, bacteriophage, CBASS, exonuclease, phage defense

1 | INTRODUCTION

Over billions of years of genetic conflict with bacteriophages and other parasitic DNA elements, bacteria have evolved myriad immunity pathways to guard themselves

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and their genomes from these assaults.[1,2] Bacterial immune systems range from the well-characterized restriction-modification and CRISPR-Cas systems to the more recently identified CBASS systems, a widespread and functionally diverse set of immune systems built around oligonucleotide second messenger signaling.[3–6] CBASS systems encode a cGAS/DncV-like nucleotidyltransferase (CD-NTase) that synthesizes one of a variety of cyclic di- and trinucleotide second messengers,[5] which activate cell-killing effectors to prevent bacteriophage replication and halt the cycle of infection.[4,7] Many CBASS systems encode proteins related to important eukaryotic signaling machinery: for example, a set of CBASS systems encoding STING-like effectors are the likely evolutionary predecessors of the mammalian cGAS-STING DNA-sensing innate immune pathway.[8] Some CBASS systems encode regulatory proteins related to eukaryotic HORMA domain and TRIP13 signaling proteins,[3,7] while others encode regulators with homology to eukaryotic ubiquitin signaling machinery.[3,9]

CBASS systems are remarkably diverse, and many systems encode putative effectors, regulators, and other ancillary proteins whose biochemical functions and biological roles remain unknown. Around 250 of the 6,233 identified CBASS systems have been reported to encode a predicted 3′-5′ exonuclease with homology to DEDDh-family DNA/RNA exonucleases.[6] This protein is found in both Type II CBASS systems that encode ubiquitin-signaling like regulators and in Type III CBASS systems that encode HORMA and TRIP13-like regulators, and is found in systems with diverse predicted effector proteins (Figure 1). The biochemical activity of this protein remains uncharacterized, and its biological role in CBASS-mediated immunity is unknown.

Here, we determine a high-resolution X-ray crystal structure of a CBASS-associated 3′-5′ exonuclease from a uropathogenic strain of *Escherichia coli* and show that it adopts a homodimeric structure similar to the bacterial DEDDh-family nucleases RNase T and Orn. We show that the protein is an active exonuclease with a preference for single-stranded DNA and does not require a CD-NTase-derived second messenger molecule for activity. Moreover, this protein is commonly found in CBASS operons that are associated with the transcription factors CapW or CapH+CapP, suggesting that the protein may regulate CBASS transcription in response to DNA damage. Together, these findings provide structural and functional insight into a previously uncharacterized enzyme associated with hundreds of bacterial CBASS immune systems.

2 | RESULTS

2.1 | Structure of *Escherichia coli* upec-117 Cap18

A recent comparative genomics study of bacteria identified and classified more than 6,200 distinct CBASS immune systems in diverse bacteria, of which around 250 were reported to encode a predicted 3′-5′ exonuclease, here termed Cap18 (Figure 1).[6,10] We manually inspected each of these systems, and found that in many cases, the noted exonuclease is encoded in a separate, neighboring operon (Tables S1 and S2). In those cases where Cap18 is encoded within the CBASS operon, we noticed that over 50% of those operons also encode transcriptional regulators of the CapW[10] or CapH+CapP[11] families. We recently showed that ~9% of CBASS systems encode either CapW or CapH+CapP and that these regulators repress CBASS transcription in uninfected cells. CapH and CapP mediate strong transcriptional activation of CBASS upon DNA damage, through direct activation of the CapP protease by single-stranded DNA.[11]

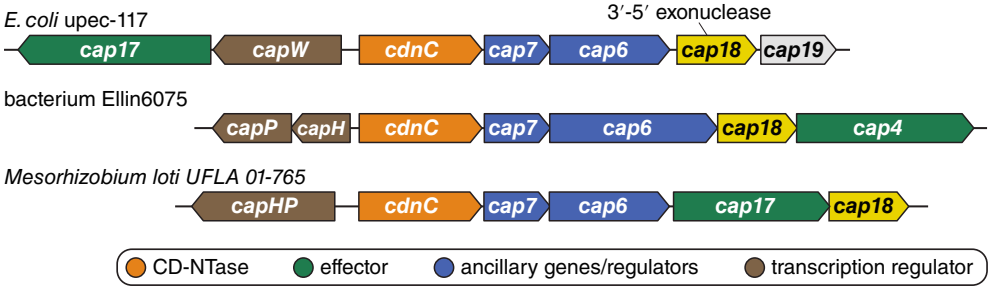

FIGURE 1 Cap18 is found in Type III CBASS systems with transcriptional regulators. Operon structure of representative bacterial CBASS systems encoding a predicted 3′-5′ exonuclease (*cap18*; yellow). cGAS/DncV-like nucleotidyl transferases (CD-NTases) are shown in orange, second messenger-activated effectors in green, and regulators in blue. CBASS-associated transcriptional regulators CapW[10] or CapH+CapP[11] are shown in brown, and proteins with unknown function are shown in gray. CapHP: fusion of CapH and CapP.[11] See Tables S1 and S2 for complete list of Cap18-encoding CBASS systems

The activating signal for CapW is unknown, but the protein's structure suggests it may also be activated by a nucleotide-based ligand or nucleic acid.[10] In this context, the high level of coincidence between Cap18 and CapW or CapH+CapP suggests that Cap18 may regulate CBASS activation through these transcription factors, by degrading free single-stranded DNA or RNA in the cell.

To determine the structure and function of Cap18, we cloned, purified, and determined a 1.82 Å resolution crystal structure of the protein from a uropathogenic strain of *E. coli*, upec-117 (Table S3). Cap18 forms a twofold symmetric homodimer (Figure 2a) with structural similarity to bacterial DEDDh-family exonucleases including RNase T[12] (Figure 2b,c, Figure S1a), Oligoribonucleotidase (Orn),[13] and RNase AS.[14] A structural overlay with substrate-bound RNase T[12] reveals close structural similarity, with an overall 1.5 Å Cα r.m.s.d. (over 133 residues) despite a low level of sequence identity at 22%. The predicted active site of Cap18 overlays closely with that of RNase T (Figure 2d,e), with the majority of active-site residues positioned for substrate- and Mg^{2+} cofactor binding: in Cap18, the exonuclease I motif comprises Asp12 and Glu14, the exonuclease II motif comprises Asp104, and the exonuclease III motif comprises Asp165. The predicted active-site histidine, His160, is positioned in a disordered loop encompassing residues 156–160 and is not resolved in our structure. This flexible loop likely requires substrate binding for proper orientation of His160.

The substrate-binding sites of bacterial DEDDh-family exonucleases are exquisitely adapted to their preferred substrates. For example, Orn is highly specific for cleaving diribonucleotides, and the preferred dinucleotide substrate fits tightly in the active site, with each base stacked against highly conserved aromatic residues in the protein.[15] RNase T, meanwhile, shows a more open active site structure consistent with its more relaxed substrate preferences.[12,15] A structure of RNase T bound to a four-nucleotide DNA substrate shows that a set of conserved aromatic residues bind and position the substrate in the active site, including Phe29, Phe77, and Phe146 (Figure 2e). Inspection of the equivalent positions in Cap18 reveals conserved hydrophobic, but not aromatic residues: Pro18, Val58, and Ile123 (Figure 2d, Figure S1a).

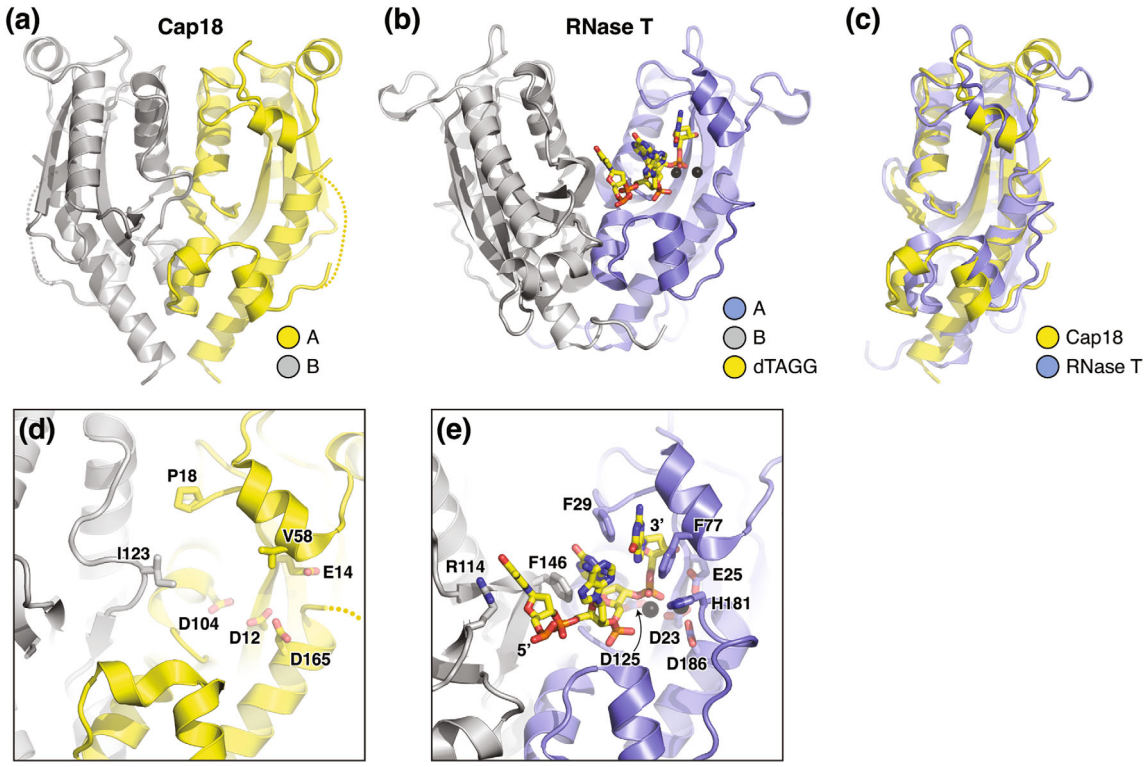

FIGURE 2 Structure of *Escherichia coli* upec-117 Cap18. (a) Structure of *E. coli* upec-117 Cap18. The two monomers of the Cap18 homodimer are shown in yellow and gray, respectively. (b) Structure of *E. coli* RNase T bound to a 5′-dTAGG-3′ DNA substrate (yellow sticks), with the two monomers colored blue and gray, respectively (PDB ID 3NH1;[12]). (c) Structural overlay of *E. coli* upec-117 Cap18 (yellow) and RNase T (PDB ID 3NH1;[12]). *E. coli* upec-117 Cap18 shows 22% overall sequence identity with RNase T and shows an overall Cα r.m.s.d. of 1.5 Å (133 residues aligned). (d) Close-up of the predicted active site of Cap18, with predicted active site residues shown as sticks. (e) Close-up of the active site of dTAGG-bound RNase T in the same orientation as panel (d), with active site residues shown as sticks and Mg^{2+} ions shown as black spheres

This observation suggests that Cap18 may show less substrate specificity than most other members of the DEDDh exonuclease family.

CBASS effector proteins, including the CapV phospholipase and the NucC and Cap4 dsDNA endonucleases, are activated by the cyclic di- or trinucleotide second messenger produced by their cognate CD-NTase.[16–18] We recently showed that *E. coli* upec-117 CBASS protects bacteria against infection by phage λ, and that the system's predicted MTA/SAH-family nucleosidase Cap17 is required for immunity, while Cap18 is dispensable for immunity.[10] These data show that Cap18 is not the primary effector of the *E. coli* upec-117 CBASS system, suggesting an accessory role for the protein. Our structure of Cap18 shows that this protein lacks a recognized second messenger binding domain, suggesting that its activity is unlikely to be modulated by second messengers produced by its cognate CD-NTase.

2.2 | Cap18 selectively degrades single-stranded DNA

DEDDh exonucleases tend to have specialized functions and substrates, often showing strong selectivity for particular structures or lengths of nucleic acid (RNA or DNA). To test the activity of Cap18, we incubated *E. coli* upec-117 Cap18 with a panel of short DNA and RNA oligonucleotides and measured its ability to cleave these substrates. We found that Cap18 is an active 3′-5′ exonuclease with a preference for ssDNA over ssRNA (Figure 3a, Figure S1b). The observed cleavage activity was low (micromolar enzyme concentrations were required to observe cleavage of nanomolar concentrations of oligonucleotide) but could be attributed to Cap18, since a catalytic-dead mutant (D165N) showed no activity (Figure 3b). We next tested a variety of double-stranded DNA substrates with 3′ overhangs, 5′ overhangs, or mismatches at one end of the cleaved strand. We found that Cap18 most efficiently cleaved substrates with 3′ overhangs or mismatches at the 3′ end, and showed nearly no activity on fully double-stranded DNA or a structure with a 5′ overhang (Figure 3b). Finally, we tested whether Cap18 shows any preferences for a particular DNA base. While the enzyme showed slightly different cleavage activity on different single-nucleotide substrates, it did readily cleave all four bases (Figure 3c).

3 | DISCUSSION

Here we show that CBASS-encoded Cap18 is a 3′-5′ DNA exonuclease with strong structural homology to the bacterial DEDDh exonucleases RNase T and Orn. The protein shows non-sequence specific activity on both single-stranded and 3′ overhang DNA substrates, and does not require a second messenger for activity. The cleavage activity we observe in vitro is very low, however, with micromolar enzyme concentrations required for measurable cleavage of nanomolar concentrations of oligonucleotide (see Methods). This observation, combined with our finding that Cap18 lacks conserved aromatic

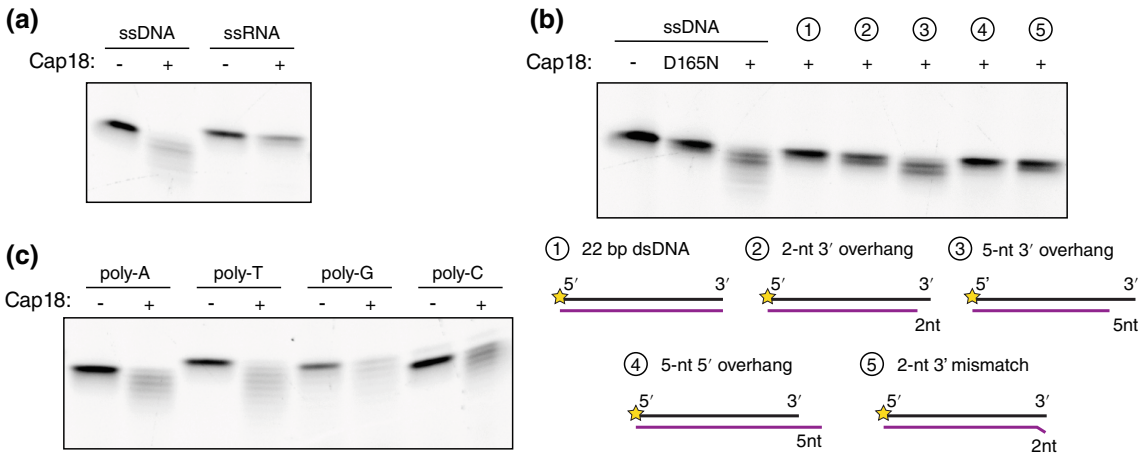

FIGURE 3 Nuclease activity of Cap18. (a) DNA exonuclease assays testing Cap18 cleavage activity on 5′ end-labeled ssDNA or ssRNA (22 bases; see Table S4 for oligonucleotide sequences), analyzed by denaturing PAGE. See Figure S1b for activity on 5′ and 3′ end-labeled ssDNA. (b) DNA exonuclease assays on wild-type or catalytic-dead (D165N) Cap18, on ssDNA and a range of double-stranded substrates. To generate dsDNA substrates, the same end-labeled oligonucleotide was annealed with different complementary strands (see diagram at bottom and Table S4). (c) DNA exonuclease assays with 22mer ssDNAs containing a single base. Since a 22mer poly-G sequence could not be synthesized, the poly-G substrate contained 16 A bases followed by six G bases (Table S4)

residues to tightly bind substrate, suggests that Cap18 may act as a slow, nonspecific DNA exonuclease.

We found that more than 50% of CBASS systems encoding Cap18 also encode transcriptional regulators of the CapW or CapH+CapP families. This high level of coincidence—only 9% of CBASS systems overall encode these regulators—suggests a functional relationship. We recently reported that CapH and CapP mediate strong transcriptional activation of CBASS upon DNA damage, through direct activation of CapP's protease activity by single-stranded DNA.[11] In this light, we propose that Cap18 may limit inappropriate activation of CBASS by degrading single-stranded DNA in the cell. This role for Cap18 is conceptually similar to CARF-type nucleases encoded by Type III CRISPR systems, which slowly degraded the cyclic oligoadenylate second messengers synthesized by these systems' effectors to prevent inappropriate activation.[19] This proposed role is also consistent with our prior finding that in the *E. coli* upec-117 CBASS system, deletion of the *cap18* gene does not affect the system's ability to protect against infection by phage λ.[10] Further work will be required to determine the functional relationship between Cap18 and the transcriptional regulation of CBASS.

4 | METHODS

4.1 | Cloning and protein purification

The coding sequence for *E. coli* upec-117 Cap18 (Joint Genome Institute IMG Gene ID 2564596480; NCBI GenBank accession # WP_001258365.1) was synthesized (GeneArt) and cloned into UC Berkeley Macrolab vector 1B (Addgene #29653) to generate an N-terminal fusion to a TEV protease-cleavable His_6-tag. Protein was expressed in *E. coli* strain Rosetta2 pLysS (EMD Millipore) by growing cultures in 2xYT media to mid-log phase at 37°C, followed by induction with 0.25 mM IPTG at 20°C for 16 hours. For protein purification, cells were harvested by centrifugation, suspended in resuspension buffer (25 mM Tris–HCl pH 7.5, 300 mM NaCl, 20 mM imidazole, 2 mM β-Mercaptoethanol and 10% glycerol) and lysed by sonication. Lysates were clarified by centrifugation (16,000 rpm/35,000 xg, 30 min), then the supernatant was loaded onto a Ni^{2+} affinity column (HisTrap HP; GE Life Sciences) pre-equilibrated with resuspension buffer. The column was washed with buffer containing 25 mM imidazole and 300 mM NaCl and eluted with a buffer containing 250 mM imidazole and 100 mM NaCl. The elution was loaded onto an anion-exchange column (Hitrap Q HP; GE Life Sciences) and eluted using a 100–800 mM NaCl gradient. Fractions containing the protein were pooled and mixed with TEV protease (1:20 protease: Cap18 by weight) plus an additional 100 mM NaCl, then incubated 16 hr at 4°C for tag cleavage. Cleavage reactions were passed over a Ni^{2+} affinity column, and the flow-through containing cleaved protein was collected and concentrated to 2 ml by ultrafiltration (Amicon Ultra-15; EMD Millipore), then passed over a size exclusion column (HiLoad Superdex 200 PG; GE Life Sciences) in a buffer containing 20 mM Tris–HCl pH 7.5, 300 mM NaCl, 10% glycerol, and 1 mM DTT. Purified proteins were concentrated by ultrafiltration and stored at 4°C for crystallization, or aliquoted and frozen at −80°C for biochemical assays. For crystallization, protein was buffer-exchanged into crystallization buffer containing 20 mM Tris–HCl pH 7.5, 100 mM NaCl, and 1 mM DTT.

4.2 | Crystallization and structure determination

We obtained crystals of *E. coli* upec-117 Cap18 in hanging drop format by mixing protein (15 mg/ml) in crystallization buffer 1:1 with well solution containing 20 mM Tris–HCl pH 7.5, 0.2 M Li Sulfate, 0.1 M Na Acetate, 0.1 M HEPES pH 7.5, and 17% PEG 4000. For cryoprotection, we added an addition 20% Glycerol, then flash-froze crystals in liquid nitrogen and collected diffraction data on beamline 24ID-C at the Advanced Light Source at Argonne National Laboratory (support statement below). We processed all datasets with the RAPD automated data-processing pipeline (https://github.com/RAPD), which uses XDS[20] for data indexing and reduction, AIMLESS[21] for scaling, and TRUNCATE[22] for conversion to structure factors. We determined the structure by single-wavelength anomalous diffraction methods with a 2.21 Å resolution dataset from crystals soaked for 1 min in cryoprotectant solution containing 0.4 M NaBr (Table S3). We identified heavy-atom sites using hkl2map[23] (implementing SHELXC and SHELXD[24]), then provided those sites to the PHENIX Autosol wizard,[25] which uses PHASER[26,27] for phase calculation and RESOLVE for density modification and automated model building.[28,29] We manually rebuilt the model in COOT,[30] followed by refinement in phenix.refine[31] using positional, individual B-factor, and TLS refinement (statistics in Table S3).

4.3 | APS NE-CAT support statement

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(P30 GM124165). The Pilatus 6M detector on 24-ID-C beam line is funded by a NIH-ORIP HEI grant (S10 RR029205). This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

4.4 | Nuclease assays

In all nuclease assays, 10 μM *E. coli* upec-117 Cap18 and 50 nM 5′6-FAM (fluorescein) labeled DNA/RNA substrate (see Table S4 for sequences) were incubated at 37°C for 30 min in Exonuclease Cleavage Buffer (20 mM Tris pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$). Subsequently, the cleavage reaction was quenched with 140 mM EDTA and incubated at 95°C for 5 min. The reaction was loaded on a pre-run 15% Novex TBE-Urea gel (Invitrogen) and run at 150 V in 1xTBE buffer. The gel was then imaged in a Bio-Rad gel imaging system. For double-stranded substrates, 1.5 μM unlabeled complementary oligonucleotides were incubated with 1 μM FAM-labeled ssDNA at 95°C for 5 min in Annealing Buffer (20 mM Tris pH 7.5, 50 mM NaCl) and slowly cooled to room temperature over 60 min.

AUTHOR CONTRIBUTIONS

Qishan Liang: Investigation (equal); visualization (equal); writing – original draft (equal). **Sara T Richey:** Investigation (equal). **Sarah N Ur:** Investigation (equal). **Qiaozhen Ye:** Investigation (equal). **Rebecca K Lau:** Investigation (equal). **Kevin Corbett:** Conceptualization (equal); funding acquisition (equal); project administration (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The 3D coordinates and structure factors for Cap18 are available at the RCSB Protein Data Bank under accession number 7T2S. Primary diffraction data for Cap18 is available at the SBGrid Data Bank under accession number 862 (native) and 863 (NaBr derivative). All other data is available upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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